NUCLEIC ACIDS ENCODING PEPTIDES HAVING THE BIOLOGICAL ACTIVITY OF SORBIN

The present invention relates to the nucleic acids encoding peptides having the biological activity of sorbin, to the peptides thus encoded and to their therapeutic applications.

Sorbin is known to be a 153 amino acid peptide of 17 500 Da, isolated and purified from pig small intestine (Vagne-Descroix et al, Eur. J. Biochem, 1991, *201*: 53-59, and patent application WO 89/06241). One of the known biological activities of this molecule consists of an increase in the absorption of water and of electrolytes (such as chloride and sodium ions) in the intestine and the gall bladder (Charpin et al, Gastroenterology, 1992, *103*: 1568-1573). Due to its properties on absorption, sorbin may advantageously be used in therapy, in particular in the treatment of diarrhea, of chronic malabsorption or of certain electrolyte disorders.

In order to produce active sorbin on a large scale, it was essential to clone the nucleotide sequence encoding this peptide. However, to date, it had not been possible to achieve this cloning due to particular difficulties linked to the structure of the peptide and to its low expression.

In fact, the active site of sorbin is located in the C-terminal part of the sequence, which is a region insensitive to the action of trypsin and chymotrypsin and to oxidation. The protein sequence of sorbin, as determined by automatic sequencing after purification, contains many amino acids encoded by degenerate codons with only 4 methionines. The cloning of a coding sequence from the corresponding known amino acid sequence requires choosing oligonucleotide primers for the polymerase chain reaction (PCR) technique. Since the degree of degeneracy of the primers is exceptionally high in the case of the cloning of

sorbin, the theoretical number of sequences able to be obtained from these primers was of the order of 6×10^{24}.

In addition, the authors of the present invention had to confront the problem of the low expression of sorbin in normal tissues, due to the very small number of endocrine cells and to the rarity of the mRNAs.

The authors of the invention have now succeeded in cloning the sorbin gene, in particular in pigs and humans.

A subject of the present invention is therefore a nucleic acid encoding a peptide having the biological activity of sorbin, said nucleic acid comprising the nucleotide sequence selected from:

a) the sequence SEQ ID No. 1;

b) the sequence SEQ ID No. 3;

c) a nucleotide sequence homologous to the sequence SEQ ID No. 1 or No. 3; and

d) at least one nucleotide fragment of said sequence a), b) or c).

The sequence SEQ ID No. 1 represents the cDNA sequence encoding porcine sorbin.

The sequence SEQ ID No. 3 represents the cDNA sequence encoding human sorbin, obtained from normal large intestine RNA by RT-PCR.

A subject of the present invention is also a nucleic acid comprising the sequence SEQ ID No. 5. This sequence represents a cDNA sequence obtained from RNA of an intestinal tumor by RT-PCR. It is, however, also present in the normal human tissue. Reference will preferentially be made to short form for the cDNA sequence SEQ ID No. 3 and to long form for the cDNA sequence SEQ ID No. 5. An unknown long fragment is inserted into the long form upstream of the amidation site and of the last six amino acids of the C-terminal region. The homologs and the fragments of this long form are also included in the present invention.

The term "homologous nucleotide sequence" is intended to mean any nucleotide sequence which differs from the sequence SEQ ID No. 1, SEQ ID No. 3 or SEQ ID No. 5 by substitution, deletion and/or insertion of a nucleotide or of a small number of nucleotides, at positions such that these homologous nucleotide sequences encode homologous polypeptides as defined hereinafter.

Preferably, such a homologous nucleotide sequence is at least 75%, preferably at least 85%, even more preferably at least 95%, identical to the sequences SEQ ID No. 1, No. 3 or No. 5.

Preferentially, such a homologous nucleotide sequence hybridizes specifically to the sequences complementary to the sequence SEQ ID No. 1, No. 3 or No. 5, under stringent conditions. The parameters which define the conditions of stringency depend on the temperature at which 50% of the paired strands separate (Tm).

For sequences comprising more than 30 bases, Tm is defined by the equation: Tm = 81.5 + 0.41 (%G+C) + 16.6 Log (cation concentration) - 0.63 (% formamide) - (600/number of bases) (Sambrook et al, Molecular Cloning, A laboratory manual, Cold Spring Harbor Laboratory Press, 1989, pages 9.54-9.62).

For sequences less than 30 bases long, Tm is defined by the equation: Tm = 4 (G+C) + 2 (A+T).

Under suitable conditions of stringency, at which the aspecific sequences do not hybridize, the hybridization temperature is approximately 5 to 30°C, preferably 5 to 10°C, below Tm, and the hybridization buffers used are preferably solutions of high ionic strength, such as a 6 x SSC solution for example.

The term "nucleotide fragment" is intended to mean any fragment of the sequence SEQ ID No. 1, SEQ ID No. 3 or SEQ ID No. 5, or of the nucleotide sequences homologous to these sequences, which encodes a peptide having the biological activity of sorbin.

The term "biological activity of sorbin" is intended to refer in particular to the known and measurable activity of sorbin on the absorption of water and of electrolytes. The activity of sorbin may in particular be measured by the decrease in weight of an isolated gall bladder filled with Krebs solution (Data for Biochemical Research, Dawson RWC, Eliott D, Eliott WH, Jones KM, Oxford at Clarendon Press, (1959)) and immersed in this nutrient Krebs solution, the decrease in weight reflecting absorption of the water content of the gall bladder. This decrease in weight is accentuated for the treated bladders compared to the controls.

The activity of sorbin may also be measured by the disappearance of electrolytes, in particular of Na^+ and Cl^- ions, from a ligatured intestinal loop *in situ* in an anesthetized rat, which is filled with a solution of known concentration. The disappearance of ions after a given time reflects the absorption of these ions from the intestinal lumen to the inside environment.

Antisecretory activity of sorbin may also be measured with the model below, during stimulation of intestinal secretion with vasoactive intestinal peptide or with cholera toxin. Sorbin in fact causes a decrease in the secretions of water and of Na^+ and Cl^- ions in this model (Marquet et al, 1994; Grishina et al, 1995; Marquet et al, 1998).

Since the biological activity of sorbin is borne by the amidated form, the nucleotide fragments of interest are therefore advantageously the fragments comprising the codons corresponding to the amidation site Gly-Arg-Arg.

Among the fragments of interest, mention may be made in particular of the nucleotide fragments comprising the sequences SEQ ID No. 6 to 8, encoding the amidated peptides of amino acid sequences SEQ ID No. 9 to 11, respectively.

The various nucleotide sequences of the invention may or may not be of artificial origin. They

may be DNA or RNA sequences obtained by screening libraries of sequences using probes developed on the basis of the sequence SEQ ID No. 1, No. 3 or No. 5. Such libraries may be prepared using conventional molecular biology techniques known to those skilled in the art.

The nucleotide sequences according to the invention may also be prepared by chemical synthesis, or using mixed methods including the chemical or enzymatic modification of sequences obtained by screening libraries.

The nucleotide sequences of the invention allow the preparation of nucleotide probes which hybridize specifically with a sequence SEQ ID No. 1, No. 3 or No. 5 according to the invention. The suitable hybridization conditions correspond to the conditions of temperature and of ionic strength usually used by those skilled in the art, preferably under stringent conditions as previously defined. Such probes are also part of the invention. They may be used as an *in vitro* diagnostic tool for detecting, via hybridization experiments, in particular "*in situ*" hybridization experiments, transcripts specific for the polypeptides of the invention in biological samples, or for detecting aberrant syntheses or genetic abnormalities resulting from a polymorphism, from mutations or from incorrect splicing.

The probes of the invention comprise a minimum of 10 nucleotides, and preferably at least 14 nucleotides, preferentially at least 20 nucleotides, even more preferentially at least 50 nucleotides, and at most comprise all of the nucleotide sequence SEQ ID No. 1, No. 3 or No. 5 or of the strands complementary thereto.

Preferentially, the probes of the invention are labeled prior to their use. For this, several techniques are within the scope of those skilled in the art, such as, for example, fluorescent, radioactive, chemiluminescent or enzymatic labeling.

The *in vitro* diagnostic methods in which these nucleotide probes are used for detecting aberrent syntheses or genetic abnormalities, such as loss of heterozygocity and gene rearrangement, in the nucleic acid sequences encoding a peptide of the invention, are included in the present invention.

A subject of the invention is also a method for detecting the expression of sorbin in a cell or tissue sample, comprising the steps consisting in:

- preparing the RNA of said sample;
- bringing said RNA obtained into contact with a probe having a nucleotide sequence capable of hybridizing specifically with a nucleic acid encoding a peptide having the biological activity of sorbin, as defined above;
- detecting the presence of mRNA which hybridizes with this probe, indicating the expression of a peptide having the biological activity of sorbin in the sample.

A subject of the invention is also a method for detecting the expression of sorbin in cells or a tissue by *in situ* hybridization, comprising the steps consisting in:

- bringing said cells or said tissue into contact with a probe having a nucleotide sequence capable of hybridizing specifically with a nucleic acid encoding a peptide having the biological activity of sorbin, as defined above;
- detecting the presence of mRNA which hybridizes with this probe, indicating the expression of the peptide having the biological activity of sorbin.

The cDNA probes of the invention can also be advantageously used to detect chromosomal abnormalities.

The nucleotide sequences of the invention are also useful for producing and using sense and/or anti-sense oligonucleotide primers for sequencing reactions or specific amplification reactions according to the "PCR" (polymerase chain reaction) technique or any other variant thereof.

The nucleotide sequences according to the invention are, moreover, of use in the therapeutic domain, for preparing antisense sequences capable of hybridizing specifically with a nucleic acid sequence, including a messenger RNA, which can be used in gene therapy. A subject of the invention is thus antisense sequences capable of inhibiting, at least partially, the production of sorbin, as defined above. Such sequences advantageously consist of those which constitute the reading frame encoding sorbin at the level of the transcript.

Among the oligonucleotide primers or probes of interest, mention may be made in particular of the oligonucleotides comprising the sequences SEQ ID No. 12 to SEQ ID No. 20 or the sequences complementary thereto.

The nucleotide sequences according to the invention may also be used to transform target cells and to make them express a peptide having the biological activity of sorbin.

A subject of the invention is therefore also a pharmaceutical composition comprising a nucleic acid according to the invention encoding a peptide having the biological activity of sorbin, in combination with a pharmaceutically acceptable vehicle, said composition being intended to be used in gene therapy. The nucleic acid of interest, preferably inserted into a vector, may be administered in naked form or in combination with at least one agent which facilitates the transfection of said nucleic acid.

Using the cDNA sequences cloned, the authors of the present invention have been able to deduce therefrom the amino acid sequence of the peptides encoded by these cDNA sequences.

The sequence SEQ ID No. 2 is the amino acid sequence of porcine sorbin.

The sequence SEQ ID No. 4 is the amino acid sequence of short form human sorbin, encoded by the nucleotide sequence SEQ ID No. 3 obtained from normal large intestine RNA by RT-PCR.

The authors of the present invention have compared the protein sequence of porcine sorbin translated from the cDNA (SEQ ID No. 2) with the sorbin sequence obtained by protein sequencing (WO 89/06241). The sorbin sequence available to date, obtained by protein sequencing (automatic or manual), proved to contain errors. The corrections are as follows: replacement W16T and replacements D35K and W112R.

A subject of the present invention is therefore a recombinant peptide having the biological activity of sorbin and comprising the amino acid sequence selected from the sequences SEQ ID No. 2, SEQ ID No. 4 and SEQ ID No. 11.

The peptides homologous to the peptides of sequence SEQ ID No. 2 or No. 4 are also included in the invention.

The term "homologous peptide" is intended to mean any peptide having an amino acid sequence which differs from the sequence SEQ ID No. 2 or SEQ ID No. 4 by substitution, deletion and/or insertion of an amino acid or of a small number of amino acids, at positions such that these modifications do not significantly harm the biological activity of sorbin. The peptide having the sequence obtained by automatic sequencing, as represented in figure 1, is excluded from this definition of the homologous peptides.

Said substitutions are preferably conservative substitutions, i.e. substitutions of amino acids of the same class, such as substitutions of amino acids with uncharged side chains (such as asparagine, glutamine, serine, threonine or tyrosine), of amino acids with basic side chains (such as lysine, arginine or histidine), of amino acids with acid side chains (such as aspartic acid or glutamic acid), or of amino acids with apolar side chains (such as glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan or cysteine).

Preferably, such a homologous amino acid sequence is at least 85%, preferably at least 95%, identical to the sequence SEQ ID No. 2 or No. 4.

Homology is generally determined using a sequence analysis program (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Similar amino acid sequences are aligned so as to obtain the maximum degree of homology (i.e. identity). To this end, it may be necessary to artificially introduce gaps into the sequence. Once the optimum alignment has been produced, the degree of homology (i.e. identity) is established by recording all the positions for which the amino acids of the two compared sequences are identical, relative to the total number of positions.

The nucleotide sequences according to the invention may, moreover, be used for producing recombinant polypeptides having the biological activity of sorbin as defined above.

These polypeptides may be produced from the nucleotide sequences defined above, according to techniques for producing recombinant products, known to those skilled in the art.

According to one embodiment of the invention, the nucleotide sequence may be inserted into an expression vector in which it is functionally linked to elements which allow the regulation of its expression, such as, in particular, transcription promoters and/or terminators.

The signals which control the expression of the nucleotide sequences (promoters, activators, termination sequences, etc.) are selected depending on the cellular host used. To this effect, the nucleotide sequences according to the invention may be inserted into vectors which replicate autonomously in the host chosen, or vectors which integrate in the host chosen. Such vectors will be prepared according to the methods commonly used by those skilled in the art, and the

clones resulting therefrom may be introduced into a suitable host using standard methods, such as, for example, electroporation or calcium phosphate precipitation.

The cloning and/or expression vectors as described above, containing one of the nucleotide sequences defined according to the invention, are also part of the present invention. Use may in particular be made of the BlueScript SKII vector (Stratagene) and the λgt 11 vector (Stratagene).

The invention is also directed toward the host cells transfected, transiently or stably, with these expression vectors. These cells may be obtained by introducing, into prokaryotic or eukaryotic host cells, a nucleotide sequence inserted into a vector as defined above, and then culturing said cells under conditions which allow the replication and/or expression of the transfected nucleotide sequence.

The host cells according to the invention can be used in a method for producing recombinant peptide having the biological activity of sorbin, said method comprising the steps consisting in:

i) inserting a nucleotide sequence as defined above into an expression vector, said nucleotide sequence being functionally linked with elements which allow the regulation of its expression;

ii) transforming a host cell with the vector thus obtained;

iii) culturing said host cell under conditions which allow the expression of said nucleotide sequence;

iv) recovering the recombinant peptide expressed;

v) optionally purifying said peptide;

vi) optionally carrying out an amidation of the peptide produced (when the host cell is a prokaryotic cell).

The study of the biological properties of sorbin has demonstrated its advantageous effect on the absorption of water, of electrolytes and of nutrients

via the mucous membranes, in particular the digestive mucous membranes.

The authors of the invention have shown that the active sequence of this peptide is specific for certain tissues - the duodenum and the jejunum in pigs, extending to the ileum and to the colon in humans and also to certain regions of the central and peripheral nervous system.

The presence of sorbin in diverse tissues means that this peptide and its fragments are considered to have a role in the cellular transport of electrolytes, and in particular of chlorine, at all levels, and in particular in the digestive tract and in the central nervous system. In the central nervous system, it is involved in behavioral disorders linked in particular to an ionic imbalance.

These advantageous properties are accompanied by considerable innocuity.

The invention is therefore directed toward pharmaceutical compositions comprising an effective amount of at least one peptide having the biological activity of sorbin as defined above, in combination with a pharmaceutically acceptable vehicle.

A pharmaceutical composition according to the invention may in particular be administered orally, parenterally, intravenously, intramuscularly, subcutaneously, percutaneously or intranasally.

The preparation of pharmaceutical compositions which contain active principles dissolved or dispersed in these compositions is well known to those skilled in the art. In general, these compositions are prepared in the form of injectable solutions or suspensions. However, they may also be in solid forms suitable for preparing solutions or suspensions extemporaneously. The preparations may also be emulsified.

The methods of administration, the doses and the pharmaceutical forms of the pharmaceutical compositions according to the invention may be determined in the usual manner by those skilled in the art, in

particular according to the criteria generally taken into account in establishing a therapeutic treatment suited to a patient, such as, for example, the age or bodyweight of the patient, the seriousness of his/her general condition, the tolerance to the treatment and the side effects noted, etc.

The pharmaceutical compositions of the invention comprising a peptide having the biological activity of sorbin, and also the pharmaceutical compositions comprising a nucleic acid encoding such a peptide, are particularly useful in:

- the treatment of infectious diarrhea and of acute toxicoses in infants, through the beneficial effect of an increase in the absorption of water and of electrolytes,
- parenteral reanimation adjuvant treatment during the reinduction of enteral feeding, during infectious diseases or surgical interventions, in particular on the digestive sphere,
- the treatment of certain conditions of chronic malabsorption, through an increase in the absorption of carbohydrates and of amino acids, linked to the increase in the absorption of water and of electrolyses,
- the treatment of certain electrolyte disorders, and in particular those of cystic fibrosis, characterized by a disorder of the reabsorption of electrolytes in the sudoriparous, salivary and bronchial glands, and in the intestine and the pancreas,
- the treatment of obesity and water overloads with substituted derivatives of the polypeptides and peptides of the invention which suppress the absorption of water, of electrolytes and of nutrients.

The presence of sorbin in nerve fibers may mean that sorbin can be described as a neurocrine peptide. The colocalization of sorbin with hormones and neurotransmitters such as serotonin corroborates the hypothesis of a neurocrine peptide factor.

The total sequence of sorbin is 153 amino acids (459 nucleotides), representing a small part of the

coding transcript, estimated to be between 6.5 and 8 Kb by Northern blot. This difference in size makes it possible to suppose that sorbin is part of a protein complex of the prepro-protein type. Such a complex exists in most endocrine systems, and in particular for "Vasoactive Intestinal Peptide", VIP.

An intracrine effect may also be attributed to it. Specifically, the internal sequence not involved in the main physiological effect of sorbin is rich in prolines and arginines. The presence of the regions highly rich in these two amino acids is currently known through its involvement in the attachment to the SH3 domains of tyrosine kinases. In this case, the internal sequence of sorbin may act as an adapter to bring the active C-terminal region close to its site of attachment or of action.

These adapters are currently being greatly sought in order to complete the mechanisms of signal transduction between the sites of attachment and their effectors.

The fact of using the C-terminal region alone (7 to 10 C-terminal amino acids) may bypass the entire signal transduction mechanism by acting directly on pumps coupled to a receptor.

A subject of the invention is also a monoclonal or polyclonal antibody directed specifically against human sorbin, or a fragment of said antibody capable of binding specifically to human sorbin.

Preferentially, the antibodies according to the invention are specific for the N-terminal end, and in particular for the portion of amino acids 40 to 45 at this N-terminal end of human sorbin.

Polyclonal antibodies can be obtained from the serum of an animal immunized against the human sorbin according to the invention, according to the usual procedures.

The monoclonal antibodies can be obtained according to the conventional method of culturing

hybridomas described by Köhler and Milstein (Nature, (1975), vol. 256, pp. 495-497).

The antibodies or antibody fragments of the invention are, for example, chimeric antibodies, humanized antibodies, Fab fragments and F(ab')2 fragments. They may also be in the form of immuno-conjugates or of labeled antibodies. For example, they may be associated with a toxin, such as diphtheria toxin, or with a radioactive product.

The antibodies thus produced may in particular be used to detect and/or assay the human sorbin in any biological sample likely to contain it.

A subject of the invention is therefore also a method for detecting and/or immunoassaying human sorbin in a biological sample, in which:

i) said biological sample is brought into contact with an antibody as defined above, labeled in a detectable manner;

ii) the formation of an antibody-human sorbin complex, indicating the presence of human sorbin in said sample, is observed.

The antibodies of the invention may therefore advantageously be used in any situation in which the expression of human sorbin must be observed.

The figures and examples below illustrate the invention without limiting the scope thereof.

LEGENDS TO THE FIGURES

- Figure 1 represents a comparison between the protein sequence of porcine sorbin translated from the cDNA (top) and the sorbin sequence obtained by protein sequencing (bottom). The degree of homology is presented between the two sequences (middle). The modifications introduced are: (W16T), (D35K), (W112R).

- Figure 2 represents a comparison between the nucleotide sequences of porcine sorbin (top) and human sorbin (short form, bottom). The various sequence variations were confirmed by sequencing on several

clones derived from various tissues of the human digestive tract.

- Figure 3 represents a comparison of the protein sequences of human sorbin (top) and porcine sorbin (bottom) translated from the cDNA.

- Figure 4 represents sections of small intestine carcinoid tumor expressing both the sorbin protein and transcripts:

a) Revelation of the cells immunoreactive to the antibody Ac 98-128 YC-17, T5. Revelation with diaminobenzidine. The positive cells are located in the peripheral layer of the tumor nodule.

b) *In situ* hybridization using the G C4-C3 antisense probe having nucleotide sequence 316-459 of porcine sorbin (nucleotides 316 to 459 of SEQ ID No. 1). Probe labeled with digoxigenin. Streptavidin-biotin revelation, chromogen AEC. There are many hybridization sites and they are observed in cells which contain sorbin.

- Figure 5 represents sections of normal human jejunum.

a) *In situ* hybridization using the G (C4-C3 antisense) probe. Probe labeled with digoxigenin. Streptavidin-biotin revelation, chromogen AEC. Some cells of the crypts hybridize with the sorbin probe. Three labeled cells are indicated with three arrows.

b) Revelation of the cells immunoreactive to the antibody Ac 93-128 YC-17, T5. Revelation, with diaminobenzidine, of the sections adjacent to those used for the *in situ* hybridization. One of the cells which hybridizes is revealed by the antibody.

c) *In situ* hybridization using the G (sense) probe as a negative control. Probe labeled with digoxigenin.

EXAMPLES

EXAMPLE 1:

Cloning of the porcine sorbin gene

MATERIALS AND METHODS

Extraction of total RNAs

The total RNAs are isolated by the guanidium thiocyanate technique (Chomczynski et al, 1987), which uses chaotropic agents which destroy all the cell structures and liberate the nuclear and cytoplasmic RNAs and the DNA, and the proteins are denatured. A purification step is necessary, either by ultracentrifugation or by phenol-chloroform extraction.

The total RNAs are extracted from frozen dry tissues. The frozen dry tissue (1 gram) is ground until it is homogenized, using an Ultraturrax machine in the presence of 7.5 ml of lysis solution.

The homogenates are deposited onto cesium chloride cushions (5.7 M and 2.4 M CsCl) and ultracentrifuged for 16 hours at 30×10^3 rpm, at 20°C in a Beckman SW41 rotor.

The translucent pellet (containing the total RNAs) is taken up in 1 ml of sterile distilled water and then precipitated with 2.5 volumes of absolute ethanol. The RNA pellet is treated with 0.1% DEPC (diethyl pyrocarbonate) at the concentration of approximately 2 $\mu g.\mu l^{-1}$.

After calculating the RNA concentration, a qualitative control of the total RNA extraction is performed by electrophoresis on a large gel of 1% agarose in a denaturing buffer, in the following way: 5 to 10 μg of total RNAs are denatured at 65°C for 5 minutes in a solution containing denaturing agents and MOPS buffer (SIGMA). The electrophoresis of the denatured RNAs is performed on a large horizontal gel of 1% agarose (containing 6% formaldehyde) in MOPS

buffer, under a voltage of 45 volts, at laboratory temperature overnight (Lehrach et al., 1977).

RT-PCR technique

Faced with the rarity of the sorbin messenger RNAs and the difficulties in obtaining them in significant amounts, the amplification technique was therefore developed. However, since the RNA cannot, itself, be used as a matrix for the PCR, a step of reverse transcription to complementary DNA was necessary.

The choice of primers was based on the presence of the least degenerate amino acids of the sorbin protein sequence, using software (OLIGO). The sequences selected are SRB1 (5') (AARGAYACNTAYAARAC) (amino acids 14 to 19) and SRB2 (3') (GGNCGYTCRTGYTGYAG) (amino acids 142 to 147) since they are weakly degenerate and give a single band by PCR and RT-PCR: other nondegenerate primers were subsequently used (table I).

Table I: Table containing the main primers used in the RT-PCRs

SEQ ID	Designation	Sequence	Orientation	Position
12	SRB1	AARGAYACNTAYAARAC	sense	42-57
13	*S1*	CGGCCGAAGGACTGGTA	sense	34-50
14	*S2*	ACAAGCCGAGATGATGAC	sense	83-99
15	*S22*	GTCTTCAACAGAAAAGCATGAC	sense	
16	SRB2	GGNCGYTCRTGYTGYAG	antisense	441-426
17	*S4*	GGATCCCAGTCATGCTT	antisense	341-325
18	*S3*	TGGATGACTTCCCAGGC	antisense	421-405
19	*S48*	GGGTCGTTCGTGCTGCAGGATGGATGA CTTCCCAGGCTCGTATTCAAA	antisense	441-394
20	*C1*	TGCTTGCGGTTTCGTGACGGG	antisense	459-439

Reverse transcription

The minimum amount of total RNA required is 1 $\mu g.ml^{-1}$.

Two 1 ml Eppendorf tubes are prepared, one of which contains the antisense primer (SRB 2) and the

other the sense primer (SRB 1). The following are introduced into each tube:

- 50 ng of purified total RNA,
- 2 µl (1/10 of the total reaction volume) of RT buffer,
- 4 µl of dNTPs (deoxyribonucleotide triphosphates),
- 1 µl of primer (50 pmol.μl^{-1}),
- sterile distilled water (qs for 18.5 µl).

The total RNA is denatured by heating the tubes for 6 min in a water bath at 70°C, and then placing them directly in ice. After centrifugation for 1 min at 13×10^3 rpm, 0.5 µl of Rnasin and 1 µl of MMLV reverse transcriptase are added to each of the tubes. The total volume of the reaction is made up to 20 µl and incubated for 1 hour at 42°C.

PCR

After the reverse transcription, the following are added to each tube:

- 8 µl of PCR buffer,
- 4 µl of dNTPs,
- 1 µl (50 pmol) of the second primer,
- 0.5 µl of Taq polymerase,
- sterile distilled water, qs for 100 µl,

and then two drops of mineral oil are added to the tubes (to avoid evaporation during the PCR reaction) and they are placed in the thermocycler, which is programmed for 35 cycles with:

- program 1: → denaturation for 10 min at 95°C,
  denaturation for 1 min,
- program 2: → hybridization for 1 min at 42°C,
  elongation for 1 min at 72°C,
- program 3: → elongation for 10 min at 72°C,
- program 4: → storage at 4°C.

Control of the RT-PCR by electrophoresis on 2% agarose gel

100 µl of chloroform are added to each tube and the mixture is stirred with a Vortex so as to emulsify and remove the mineral oil. After centrifugation for 2 min at 13×10^3 rpm, the cDNA is recovered from each tube and the amplification is controlled on a 2% agarose gel, with a 20 min migration time at 100 volts, and the gel is observed under a 254 nm ultraviolet radiation lamp.

Subcloning technique

The subcloning technique made it possible to isolate and amplify a DNA fragment, in a monoclonal fashion, in a very large amount. Three main steps were used:

- isolation of the DNA to be inserted,
- *in vitro* recombination with a vector,
- introduction of the recombined vector into a competent host cell.

Preparation of the cloning vector

The cloning vector used is the Bluescript II SK +/- plasmid (Stratagene). The technique for preparing the vector comprises several steps:

* step 1: heterodigestion with the Eco RI and Hind III restriction enzymes for the purpose of linearizing the vector for the recombination.
* step 2: purification.
* step 3: dephosphorylation of the 5' ends via the action of a phosphatase, this being so as to avoid the vector religating with itself.
* step 4: purification.
* step 5: control of the dephosphorylation by bacterial transformation on agar plus antibiotic. If the vector is correctly dephosphorylated, there is no plasmid expression and no bacterium grows.

Preparation of the insert

The insert used is the cDNA obtained by RT-PCR and isolated by electrophoresis on 2% agarose gel.

Purification of the insert using the GeneClean *Kit* (Ozyme).

The agarose gel is delicately cut up, under the ultraviolet (254 nm) lamp, with clean scalpels for each band, and one band per Eppendorf tube (of 1.5 ml) is recovered.

- 2 volumes of NaI solution are added and the mixture is placed in a water bath at 55°C for 5 min. The use of NaI makes it possible to dissolve the agarose, to increase the ionic strength and, thus, to recover the cDNA trapped by attachment of the Na^+ ions to the phosphates of the molecule.

- 10 µl of Glass Milk (Ozyme) (small fragments of silica to which the cDNA attaches via hydrogen bonds) are added and left to act for 5 min at laboratory temperature.

- The mixture is centrifuged for 30 seconds at 13×10^3 rpm, the supernatant is removed and three successive washes are performed with the NEW Wash washing solution (Ozyme):

- 1 ml of NEW Wash is added to each tube,

wash I:
- the mixture is centrifuged for 2 min at 13×10^3 rpm,
- the supernatant is removed,

- 500 µl of NEW Wash are added,

wash II:
- the mixture is centrifuged for 2 min at 13×10^3 rpm,
- the supernatant is removed,

wash III: - identical to wash II.

The pellet containing the cDNA is dried, the dry cDNA is taken up with 50 µl of sterile distilled water and the Eppendorf tubes are placed in a water

bath at 55°C for 5 min. The role of the distilled water is to detach the cDNA attached to the silica fragments since there is no ionic strength, and the heat makes it possible to decrease the strength of the hydrogen bonds between the cDNA and the Glass Milk. Thus, the cDNA is released.

- The tubes are vortexed and centrifuged for 1 min at 13×10^3 rpm, and the cDNA is recovered for heterodigestion.

- Heterodigestion with the Eco RI and Hind III restriction enzymes.

The following are added to each tube:

- 10 µl of buffer B (restriction enzyme buffer),
- 2 µl of Eco RI enzyme (at 20 units),
- 2 µl of Hind III enzyme (at 20 units),
- sterile distilled water, qs for 100 µl of total reaction volume,

and the mixture is incubated overnight in a water bath at 37°C.

After purification once again using the GeneClean Kit, the same procedure starting from the addition of NaI is performed.

The heterodigestion is controlled by electrophoresis on 2% agarose gel and the insert is ligated with the BlueScript plasmid (*in vitro* recombination).

The following are introduced into each tube:

- (calculated volume) µl of cDNA,
- 1 µl of linearized and dephosphorylated BlueScript plasmid (50 ng),
- 1 µl of T4 DNA ligase buffer (at -20°C),
- 0.5 µl of T4 DNA ligase enzyme,
- sterile distilled water, qs for 20 µl of total reaction volume,

and the mixture is left overnight in a water bath at 16°C.

Preparation of competent bacteria

The bacterial strain used is: *Escherichia coli* strain HB101.

a) Preculture

A drop of a bacterial strain from frozen culture is taken using a Pasteur pipette equipped with a sterile tip, under a laminar flow hood, and a 4 ml tube of L. Broth culture medium (LB, Gibco-BRL) is seeded. The mixture is incubated overnight at 37°C with shaking.

b) Culture

500 µl of preculture are seeded, under the hood, into a 250 ml Erlenmeyer flask containing LB culture medium and the mixture is incubated for 2 hours 30 minutes in an incubator at 37°C with shaking. The bacterial growth is regularly controlled by measuring the OD at 600 nm (approximately every 30 min) since this OD must not exceed the value of 0.3 which corresponds to the exponential growth phase. (Generally, this value is reached after culturing for 2 hours 30 minutes.)

- When the OD = 0.3, the bacterial culture is stopped by taking the Erlenmeyer flask out of the incubator and placing it in ice. In fact, beyond this OD, the bacterial cells enter into a stationary phase which is no longer favorable to transformation due to toxin secretions and to cell death. The mixture is then centrifuged for 15 min at 3000 rpm at 4°C so as to obtain a bacterial pellet.

c) Treatment of the bacterial wall

The supernatant is removed by inverting the tube and the following are added to the pellet:

- 20% of a solution of iced Tris $CaCl_2$ (pre-filtered). The tube is placed in ice for 20 min.

- The mixture is centrifuged for 15 min at 3000 rpm at 4°C, the supernatant is removed and 10% of Tris $CaCl_2$ are added. The tube is placed at 4°C.

Bacterial transformation

5-ml sterile tubes containing the following are prepared under the hood:

- 30 µl of competent bacteria,
- 10 µl of recombinant plasmids,

and the tubes are placed in ice for 20 min.

- A heat shock is applied by placing the tubes in a water bath at 42°C for 2 minutes.
- 500 µl of LB medium are added per tube and the mixture is placed in a water bath at 37°C for 1 hour.
- 4 ml of molten soft agar medium (LB from Gibco-BRL and 7 g/1000 ml agar from Difco-USA) are added per tube and each tube is plated out onto a Petri dish containing agar, seeded beforehand with ampicillin.

Analysis of the recombinant clones

10 colonies per dish are taken, under the laminar flow hood, and are seeded into 4 ml of LB medium (to which 40 µl of 100x concentrated ampicillin have been added beforehand). The mixture is left to incubate overnight in an incubator at 37°C.

Performing a microamplification

1 : Phenol extraction

- The content of each tube amplified is transferred into Eppendorf tubes which are centrifuged for 2 min at 13 × 10^3 rpm to precipitate the bacteria.
- The supernatant is aspirated with a Pasteur pipette connected to a vacuum pump and 100 µl of phenol are added per tube, followed by vortexing. This treatment allows the extraction of all the nucleic acids (bacterial DNA and RNA and recombinant DNA) by denaturation of the proteins with the phenol.
- 100 µl of TNE (Tris Natrium EDTA, the Tris originating from Boehringer, the sodium NaCl from Merck and the EDTA from Merck), and the mixture is vortexed for 5 min and centrifuged for 2 min at 13 × 10^3 rpm. The EDTA makes it possible to inhibit the DNases and the

sodium makes it possible to have an ionic strength, and together this thus ensures the conservation of the nucleic acids.

2 : Ethanol precipitation

- 80 µl of supernatant from each tube are taken up into new Eppendorfs and 2 volumes of absolute ethanol at -20°C are added. The mixture is left for 5 min at -80°C.

- The mixture is centrifuged for 10 min at 4°C at 13×10^3 rpm, the supernatant is removed and the recombinant DNA pellet is dried.

3 : Heterodigestion of the recombinant DNA with the Eco RI and Hind III restriction enzymes

The recombinant DNA pellet is taken up with 50 µl of sterile distilled water and Eppendorf tubes are prepared.

The following are added to each tube:

- 10 µl of DNA,
- 2 µl of buffer B (restriction enzyme buffer),
- 1 µl of Eco RI enzyme (10 units),
- 1 µl of Hind III enzyme (10 units),
- sterile distilled water, qs for 20 µl,

and the mixture is incubated in a water bath at 37°C for 1 hour.

4 : Control of the heterodigestion by electrophoresis on 2% agarose gel

2 µl of loading buffer are added to each tube and the gel is loaded. Migration time: 30 minutes at 100 volts.

Performing a macroamplification

Each positive minipreparation tube is seeded, under the hood, into 1 l Erlenmeyer flasks containing:

- 200 ml of LB culture medium,
- 2 ml of 100x ampicillin,
- 5 drops from the minipreparation tube,

and incubated overnight at 37°C with shaking.

1 : Digestion of the bacterial wall

- The content of each Erlenmeyer flask is transferred into jars specific for the refrigerating centrifuge and is centrifuged for 15 min at 4000 rpm at 4°C. The supernatant is removed, 2 ml of solution I (containing lysozyme) are added per jar and, after homogenizing, this is left to act for 5 min at laboratory temperature.

- 4 ml of solution II are added per jar, and this is stirred to obtain a viscous consistency and left to act for 5 min in ice.

- 3 ml of solution III are added per jar and, after homogenizing, this is left to act for 15 min in ice.

- After centrifugation for 15 min at 8000 rpm at 4°C, the supernatant from each jar is filtered and transferred into glass tubes.

2 : Alcohol precipitation

- 4.5 ml of 2-propanol are added per tube and left to stand for 30 min at -80°C.

- The mixture is centrifuged for 20 min at 12 500 rpm at 4°C (taking care to place the tubes in protective rubber reducers), the supernatant is removed and the tubes are drained on filter paper.

- The tubes are dried to remove any traces of alcohol.

3 : Enzymatic digestion

- The DNA pellets are taken up with 3 ml of distilled water.

- RNase: 10 μl of Rnase are added per tube and left to act for 30 min in a water bath at 37°C. In this instance, the RNase enables the bacterial RNA to be digested.

- Proteinase K: 10 μl of proteinase K are added per tube and left to act for 30 min in a water bath at

37°C. This enzymatic reaction enables all the proteins to be digested.

4 : Cesium chloride gradient ultracentrifugation

The following are added to each tube:

- 3.75 g of CsCl (cesium chloride),
- 1 µl of ETB (ethidium bromide),

and the tubes are ultracentrifuged at 50 × 10^3 rpm at 20°C overnight. A pink band of DNA is then obtained which is visible under ultraviolet light.

- The pink band of recombinant DNA is carefully recovered (in 10 ml tubes) using a syringe, by inserting the needle below the band and gently aspirating. A volume of sterile distilled water is added per tube and the volume is made up to 10 ml with isoamyl alcohol. The mixture is homogenized by vigorously inverting for 3 min.

- After centrifugation for 5 min at 3000 rpm at 15°C (in order to remove all the ETB with the isoamyl alcohol) the recombinant plasmids are recovered with a Pasteur pipette equipped with a bulb (one pipette per tube is used).

5 : Ethanol precipitation

- 2 volumes of 100° ethanol are added and left to act for 30 min at -80°C (tubes have a milky appearance).

6 : Washes

- After centrifugation for 15 min at 12 500 rpm at 4°C to separate the salt from the recombinant DNA, the supernatant is discarded and 2 ml of sterile distilled water are added to eliminate the CsCl.

- 2 volumes of cold absolute ethanol (-20°C) are added to precipitate the DNA and left to act for 30 min at -80°C.

- After centrifugation for 15 min at 4°C at 12 500 rpm, the supernatant is removed and the tubes are drained on filter paper.

- The tubes are dried for 10 min in a speed vac in order to remove any traces of ethanol, and the dry pellets are taken up with 100 µl of sterile distilled water.

7 : Assaying of the DNA in a spectrophotometer at 260 nm

8 : Sequencing according to the modified Sanger method

Eppendorf tubes are prepared as indicated below:

- 5 µl of cDNA (insert at 1 $\mu g.\mu l^{-1}$)

(volume adjusted depending on the spectrophotometric assay),

- 2 µl of 2N NaOH,
- sterile distilled water, qs for 20 µl,

and left to stand for 20 min at laboratory temperature.

The mixture is neutralized with sodium acetate and ethanol precipitation is then carried out:

75 µl of cold absolute ethanol (-20°C) are added, left to act for 20 min at -80°C and centrifuged at 13 x 10^3 rpm. The tubes are dried to eliminate any trace of ethanol.

Hybridization:

The following are added to each tube:

- 2 µl of hybridization buffer (5X),
- 0.5 µl of primer (10 pmol),
- 7.5 µl of sterile distilled water,

and the mixture is left to stand for 20 min in a water bath at 37°C.

Priming the polymerization reaction:

The following are added:

- 1 µl of DDT,
- 2 µl of hybridization buffer ("GTP labeling mix", USB/Amersham (diluted 5X)),

- 0.5 µl of $^{\alpha 32}$P-dATP,
- 1.5 µl of sequenase (diluted 6-fold),

and the mixture is homogenized and left to act for 5 min at laboratory temperature.

- 3.5 µl of this mixture are transferred into 4 Eppendorf tubes each containing a different ddNTP (dideoxyribonucleotide) (4 different reactions) and this is left to act for 5 min in a water bath at 37°C. In this instance, the elongation of the chains is stopped when the ddNTPs are incorporated since the latter cannot form phosphodiester bonds with the subsequent dNTPs.

The reaction is stopped by adding 5 µl of "stop" solution (containing a colorant and formamide (Amersham)) per Eppendorf tube and the tubes are immediately placed in ice.

Loading the polyacrylamide gel

The tubes are heated to 80°C and then immediately placed in ice. The gel is loaded with deposits of 2 to 4 µl per well. For fragments of 100 bp, migration is allowed to continue for 1 hour 20 minutes to 3 hours at 1450 volts.

Termination of electrophoresis, exposure and developing by autoradiography

a) Termination of electrophoresis

The electrodes are disconnected, the migration buffer is eliminated and the glass plates containing the sequence gel are removed. The gel is recovered and a sheet of 3M Watman® paper is placed on it, patting so as to make it adhere properly, and the gel attached to the paper is deposited on a sheet of plastic film (Cellofray®) in order to protect it. The sequence gel is dried for 2 hours at 80°C under vacuum.

b) Exposure of the gel on an autoradiographic film

After drying, the Cellofray® is removed and an autoradiographic film (Hyper film-MP®, Amersham) is applied. The entire combination is then placed in a cassette (equipped with an intensifying screen) for an exposure of overnight to several days, at -80°C, since the films are more sensitive in the cold.

c) Developing of the autoradiographic film

The developing is carried out in red light and consists in passing the autoradiographic film through various baths:

- 2 min in the developer diluted to 1/5 (ILFORD 2000 RT),
- rinsing in water,
- 5 min in the fixer diluted to 1/5 (ILFORD 2000 RT),
- rinsing for 15 min in running water,
- drying and reading the film.

Preparing and labeling the probes

Labeling a probe

To detect the presence of a complementary sequence, by hybridization, in a mixture of DNA fragments, the denatured DNA probes are radioactively labeled with $^{\alpha 32}$P-dCTP (under the conditions recommended by the supplier), using the random priming technique. The probes obtained have a specific activity of 0.2 to 1×10^9 cpm.μg^{-1}.

30 ng of pure cDNA fragments (probe) in a final volume of 45 μl of sterile distilled water are denatured for 2 min at 80°C, and the entire volume is placed directly in ice.

- 45 μl of denatured probe are then introduced into a lyophilizate (Rediprime DNA kit, USB) containing:
  - the Klenow polymerase buffer,
  - the dNTPs (minus the dCTP),

- the synthetic octanucleotide primers,
- the Klenow polymerase,

and 5 µl of $^{\alpha 32}$P-dCTP radioactivity are added. The mixture is incubated for 10 min in a water bath at 37°C for polymerization according to the random priming principle.

Molecular hybridization of the labeled probes with a membrane

Northern blot and Southern blot

Northern blot is a technique for detecting a transcript in a complex mixture. The size of the RNA may be determined by the degree of its migration in the gel and its abundance may be determined by the intensity of the band (or signal). This method is widely used to study abnormalities in the transcription of a gene. It is carried out in several steps:

- transfer of the electrophoretic profile onto a nylon membrane,
- prehybridization,
- hybridization,
- washing,
- developing by autoradiography.

Transfer

After electrophoresis performed with 5 to 10 µg of denatured total RNA (on a denaturing 1% agarose gel), this is transferred onto a nylon filter (Hybond N Amersham) in the presence of phosphate buffer. The transfer takes place overnight at laboratory temperature and occurs due to the phenomenon of capillarity.

Prehybridization and hybridization

The membrane is dried for 3 hours at 80°C, before hybridization, so as to irreversibly attach the DNA.

10 ml of hybridization solution with 150 µl of denatured (2 min at 80°C) salmon sperm DNA are

introduced into a sealed plastic bag (containing the membrane). The bag is incubated for 3 to 6 hours at 42°C with shaking.

- Hybridization: The bag is carefully opened and 50 µl of denatured (2 min at 80°C) probe labeled with $^{\alpha 32}$P-dCTP are introduced. This is incubated overnight in an incubator at 42°C with shaking.

Washes

After hybridization, the mixture is removed from the plastic bag and the filter is washed in various baths:

- 1 rapid rinse in a solution of 2 x SSC at room temperature,

- 1 rinse in a solution of 2 x SSC at 65°C for 45 min,

- 1 rinse for 45 min at 65°C with shaking in a solution of 2 x SSC + 0.5% SDS,

- 1 rinse for 45 min at 65°C with shaking in a solution of 0.2 x SSC + 0.5% SDS.

Developing by autoradiography

The filter is then dried and exposed for autoradiography on film (Hyper film-MP®, Amersham) in a cassette with an intensifying screen. The exposure lasts several hours to several days at -80°C.

RESULTS

The authors of the present invention screened the cDNA expression library concerning pig duodenum and jejunum with an anti-sorbin antiserum. This screening did not make it possible to continue the search in this library. On the other hand, RT-PCR amplification in the presence of degenerate primers in the least degenerate regions proved to be positive, giving several fragments amplified from jejunum and duodenum RNA. All these fragments were cloned, sequenced and compared with the databanks. The authors of the present invention varied the various PCR parameters so as to optimize the

specificity of the primers. This made it possible to amplify a part of the sorbin sequence. The sorbin fragment confirmed by sequencing was then used as a probe to screen the expression library prepared from the same RNAs. Several clones were purified and sequenced.

The cloning of the sorbin cDNA made it possible to rectify the sorbin protein sequence in its N-terminal region (fig. 1).

EXAMPLE 2:

Cloning of the human sorbin gene

MATERIALS AND METHODS

The PCR conditions used are identical to those described in example 1.

The human sorbin gene was isolated using a probe prepared from the complete cDNA (459 base pairs) encoding porcine sorbin.

First, immunohistochemistry and *in situ* hybridization studies were carried out.

Immunohistochemistry

The protocol used is similar to that described in the article by Fatima Abou El Fadil, 1997.

The antiserum used, designated 93-I28 Y C17, was produced by inoculating a rabbit with the peptide containing amino acids 137 to 153 of porcine sorbin to which a tyrosine has been added at position 1, as described in the abovementioned article.

***In situ* hybridization**

I - Principle of *in situ* hybridization

In situ hybridization makes it possible to visualize an mRNA (transcript of a gene) at the cellular level, either on histological sections or on cell suspensions, using a labeled probe (hot-labeled or cold

labeled). Specifically, this technique is based on the property of specific pairing (with high affinity) of two complementary nucleotide sequences. It is carried out in 6 steps:

* labeling of the probe,
* pretreatment,
* hybridization,
* washes,
* developing,
* observations under an optical microscope.

II - Aim of its use

The value of its use in the case of the study of sorbin is to localize, at the cellular level, the mRNA corresponding to sorbin on histological sections of pancreatic vipoma, of healthy pancreas and of intestinal carcinoid and, by the same token, to confirm the nucleic acid and immunocytological labeling obtained in parallel. For this, probes labeled with digoxigenin-11-dUTP according to the random priming method are used.

III - Technique

III-1 - Labeling of probes with digoxigenin

This is performed using the random priming method with the Rediprime DNA kit. The cold probes are labeled with dUTP-digoxigenin.

III-2 - Preparation of slides

- The slides are washed with tap water and are then immersed overnight in hydrochloric alcohol.

- They are washed in running water for 3 to 4 hours, rinsed with distilled water and dried in an incubator at 40°C.

- A drop of poly-L-lysine (at 1% diluted in distilled water) is spread out over each slide using another slide like a smear. The slides are air-dried

(protected against dust) and incubated overnight in an incubator at 60°C.

III-3 - Preparation of sections

a) Treatment of the block

Serial sections of healthy pancreas, vipoma and intestinal carcinoid are prepared. They are cut at 3-4 μm on a microtome with a disposable knife. They are then collected on the clean slides treated with poly-L-lysine, by depositing a drop of sterile distilled water at the surface thereof. The excess water is then drained, followed by drying for 1 hour on a hot plate. The slides are incubated overnight in an incubator at 60°C. Subsequently, they may either be treated or conserved in a holder wrapped in aluminum foil and placed at 37°C.

b) Dewaxing and rehydration of sections

Aim: To remove the embedding medium from the tissue.

Technique: The sections are immersed in baths of alcohol of increasing degree:

- 3 times 5 min in xylene,
- twice 2 min in 100° ethanol,
- twice 2 min in 95° ethanol,
- rehydrated twice 5 min in PBS (phosphate buffered saline at 150 mmol.l^{-1}).

c) Pretreatments

These serve essentially to improve the signal/background noise ratio and the hybridization response. A certain number thereof exist, including:

* Chemical hydrolysis

Aim: It allows the DNA to be denatured in order to make the mRNA more accessible to the probe.

Technique: The slides are soaked in a water bath of 0.2N HCl for 12 min and rinsed twice 5 min in PBS.

* Enzymatic hydrolysis with proteinase K

Aim: This treatment allows the digestion of all the proteins, which then leads to permeabilization of the material and increases the penetration of the probe into the tissue.

Technique: The slides are soaked in 100 ml of proteinase K (at 10 $\mu g.ml^{-1}$ in Tris EDTA, pH 7.4) for 10 min at 37°C.

* Blocking of endogenous peroxydases

Aim: To avoid the interaction of tissue peroxydases with the use of biotinylated streptavidin-peroxydase, during development.

Technique: The slides are immersed in H_2O_2 (3% in PBS) for 5 min and rinsed twice 5 min in PBS.

d) Control with RNases

Aim: This involves carrying out a negative control which will make it possible to affirm, when studying the results, that the signal observed on the slides does not correspond to an artifact.

Technique: The slides are immersed in 100 ml of RNases (at 100 $\mu g.ml^{-1}$ in 2XSSC) for 30 min at 37°C and washed in 2XSSC for 15 min at room temperature.

e) Dehydration

Technique: The sections are passed through baths of alcohol of increasing degree:

- 1 bath of 70° ethanol for 1 min,
- 1 bath of 80° ethanol for 1 min,
- 1 bath of 100° ethanol for 10 min,
- drying in the open air.

III-4 - Hybridization

a) - Denaturation of the probe

Aim: This step is essential in order to have single-stranded probes, since these probes obtained by

genetic engineering are still double-stranded (and cannot therefore be used for hybridization).

Technique: The probe is added to 20 µl of hybridization solution (prepared extemporaneously) so as to obtain 10 $ng.ml^{-1}$, it is placed at 75°C for 5 min and is immediately immersed in ice.

Comment:

* The concentration of the probe may be adjusted as a function of the length. For probes of less than 1 Kb, the concentration may reach 20 $\mu g.ml^{-1}$.

b) Hybridization

Technique: The 20 µl of the denatured probe are deposited on the slide. A coverslip is mounted and the assembly is left to incubate in a humid chamber at 42°C for 16 hours, protected against dust.

III-5 - Washes

Aim: These are used to remove the excess probe, the partial hybrids and the aspecific hybrids. For this, the sections undergo washes under conditions of increasing stringency (i.e. decreasing concentration of salts and increase in temperature) which will ensure the dissociation of the partial hybrids and nonspecific hybrids.

Technique: The slides are immersed in successive baths:

- 1 bath of 4XSSC (which makes it possible to detach the cover slips),
- 1 bath of 1XSSC for 15 min at room temperature,
- 1 bath of 1XSSC for 30 min at room temperature,
- 1 bath of 1XSSC for 15 min at 42°C,
- 1 bath of 1XSSC for 30 min at 42°C,
- 1 bath of 0.1XSSC for 30 min at 42°C.

III-6 - Developing

This is an indirect method which uses immuno-cytological methods based on antigen-antibody or ligand-antiligand reactions.

a) Saturation of the endogenous biotin sites in the tissues

Aim: These sites are saturated in order to avoid attachment of the exogenous streptavidin during developing. For this, milk proteins are used.

Technique: STMT buffer (sodium-tris-magnesium-Tween, the magnesium coming from Sodipro and the Tween from Sigma) is extemporaneously prepared at 1 mol.l^{-1} with 1% of skimmed milk (pH 7.5), and rinsing is carried out:

- 1 rinse for one hour in a water bath at 37°C,
- 1 rinse for 15 min at room temperature.

b) Detection of the digoxigenin

Technique: The following are deposited onto each section:

- 200 µl of normal goat serum (diluted to 1/20 in TBS from Sigma), which is left for 30 min at room temperature in a humid chamber (protected against dust).
- 200 µl of anti-digoxigenin monoclonal antibody (diluted to 1/30 in TBS + 1% of 30% BSA), which is left for 30 min at room temperature in a humid chamber.
- Rinsing is carried out twice 5 min in TBS.
- 200 µl of biotinylated anti-mouse goat serum (DAKO kit), which is left for 30 min at room temperature in a humid chamber.
- Rinsing is carried out twice 5 min in TBS.
- A few drops of biotinylated streptavidin-peroxydase are added and left for 30 min at laboratory temperature in a humid chamber.
- The peroxydase activity is developed with the DAKO AEC red chromogen kit, in the dark.

- Counter-staining with hemalun may be carried out.

c) Controls

A signal observed on a slide needs to be verified with other control slides in order to affirm the specificity of the response.

Thus, depending on the treatments performed on the slides, the following are produced:

- treatment in the absence of probe: the labeling is linked to the presence of endogenous biotin.

- treatment in the presence of RNAse: no *in situ* hybridization.

RESULTS

Initially, sorbin was sought in gastrointestinal and pancreatic tumors by immunohistochemistry with antibodies specific for the C-terminal active region of sorbin. Tumors which were positive by immunohistochemistry were used in RT-PCR. After extraction of the RNAs and amplification, the presence of sorbin was confirmed by the presence of a band with the expected size and by sequencing. The fragments which were considered to be background interference and which did not correspond to the correct size were also sequenced. Their sequences did not correspond to sorbin and had no homology in the databanks.

The *in situ* hybridization with probes for the C-terminal region shows that only some endocrine cells express this peptide in the normal state, as well as the peripheral cell layers of certain intestinal and pancreatic tumors.

The immunohistochemistry and *in situ* hybridization studies carried out on the same histological sections of the same tumors showed a very strong correlation for the endocrine cells expressing sorbin (figure 4).

The same correlation between the results obtained with the two techniques was found in normal human jejunum (figure 5).

Two forms were, in fact, obtained by RT-PCR:

- a short form close to porcine sorbin was obtained by RT-PCR on a normal human large intestine;

- a long form with a central region different from porcine sorbin was obtained by RT-PCR both in a gastrointestinal tumor tissue and in a normal tissue.

The protein sequences of the short human sorbin and of the porcine sorbin, translated from the cDNAs, were compared (figure 3).

REFERENCES

Abou El Fadil F., Sorbin in the porcine gastro-intestinal tract and pancreas: an immunocytochemical analysis. *Endocrinology*, vol 138, No. 11, 1997.

Charpin G., Chikh-Issa AR., Guignard H., Jourdan G., Dumas C., Pansu D., Descroix-Vagne M, Effect of sorbin on duodenal absorption of water and electrolytes in the rat. *Gastroenterology* 103: 1568-1573, 1992.

Chomczynski P. and Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162: 156-159, 1987.

Eto B., Griesmar B., Desjeux JF. Effect of sorbin on electrolyte transport in rat and human intestine. *Am. J. Physiol.*, 1999; 276 (*Gastrointest. Liver. Physiol.* 39): G107-G114.

Grishina O., Charpin G., Marquet P., Pansu D., Descroix-Vagne M. Effect des dérivés C-terminaux de la sorbine sur les flux ioniques duodénaux chez le rat. *Gastroenterol. Clin. Biol.*, 1995: 19: 487-93.

Lehrach H., Diamon D., Wozney J.M. and Boedker H. RNA molecular weight determination by gel electro-phoresis under denaturing conditions: a critical reexamination. *Biochemistry*. 16: 4743-4751, 1977.

Marquet F., Grishina O., Pansu D., Descroix-Vagne M., Effet des dérivés C-terminaux de la sorbine sur les flux ioniques ileaux stimulés par le VIP chex le rat. *Gastroenterol Clin. Biol.*, 1994; 18: 702-707.

Marquet F., Botella A., Bueno L., Pansu D., Decroix-Vagne M. Effect of sorbin derivatives on

cholera toxin-induced intestinal secretion in rat *in vivo*. *Peptides* 1998: 19: 1417-1423.

Vagne-Descroix M., Pansu D., Jörnvall H., Carlquist M., Guignard H., Jourdan G., Desvigne A., Collinet M., Caillet C., Mutt V., Isolation and characterisation of porcine sorbin, *Eur. J. Biochem.* 201: 53-59, 1991.